

PCR and In Vitro Cultivation for Detection of *Leishmania* spp. in Diagnostic Samples from Humans and Dogs

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A PCR assay for the diagnosis of leishmaniosis was developed by using primers that were selected from the sequence of the small-subunit rRNA gene. The assay was optimized for routine diagnostic use. Processing of the clinical samples is rapid and simple (lysis of erythrocytes in Tris-EDTA buffer, digestion with proteinase K directly in PCR buffer, and no further purification steps). Furthermore, an internal control is included in every specimen in order to detect the presence of PCR inhibitors. The PCR was compared with diagnostic in vitro cultivation of promastigote stages for the detection of *Leishmania* spp. in clinical specimens from humans and dogs with a tentative diagnosis of leishmaniosis. PCR and cultivation gave identical results with all but 1 of the 95 specimens from humans. The PCR result in this case was false negative, possibly because of unequal apportionment of this sample. With 10 skin biopsies from six patients with cutaneous leishmaniosis, the sensitivity was 60%. For six human immunodeficiency virus-positive patients with visceral leishmaniosis, all bone marrow biopsies and 7 of 11 whole blood samples (after isolation of leukocytes by Ficoll-Paque) were positive in both tests. PCR detected one more case with the use of 500 μ l of whole blood with direct lysis of the erythrocytes in Tris-EDTA buffer. With dog lymph node aspirates, the sensitivity was 100% (16 of 16 samples) for both methods; furthermore, PCR was positive for 5 of 13 whole blood samples from dogs with leishmaniosis. The specificity of the PCR was 100% (70 specimens from patients without leishmaniosis). This PCR assay proved to be feasible as a routine diagnostic test, being reliable and faster than in vitro cultivation.

Various species of the genus *Leishmania* are the causative agents of visceral, cutaneous, and mucocutaneous leishmaniosis in humans and of leishmaniosis in dogs. The incidence of cases in humans has been reported to be increasing. Causes for the increase are, among others, the spread of the parasites to new geographical areas (10, 21, 34) and the fact that visceral leishmaniosis is an opportunistic infection in immunocompromised patients (11, 17), thereby changing the picture of visceral leishmaniosis as being predominantly a children's disease (2, 3, 20).

With increasing international travel, cases of imported leishmaniosis are regularly diagnosed in both humans and dogs in areas where the disease is not endemic. Diagnosis can be achieved either by demonstrating the parasite microscopically (in stained smears or after infection of hamsters or in vitro cultivation) or indirectly by serological means. Visceral leishmaniosis, caused by *L. donovani*, *L. infantum*, and *L. chagasi*, is routinely diagnosed by enzyme-linked immunosorbent assay (ELISA) in immunocompetent patients. However, the sensitivity of serological methods when used with AIDS patients was reported to vary in the range from 6 to 82% (13, 15, 24, 26, 27). This variability may partially result from the different techniques used. The sensitivity of antibody detection in such patients with leishmaniosis was improved by using more than one test system and especially by using Western blot (immunoblot) analysis (13). Furthermore, it was suggested that human immunodeficiency virus (HIV)-positive patients without specific anti-*Leishmania* antibodies could be those who were infected with *L. infantum* only after immunodepression had developed (15). Other drawbacks of serological methods are that they cannot be used when one attempts to monitor pa-

tients during chemotherapy and that they fail to diagnose relapses, which are common in AIDS patients even when they have been correctly treated (3, 27).

Diagnosis in patients with localized cutaneous leishmaniosis (25) caused by *L. major*, *L. tropica*, and species of the *L. braziliensis*-*L. mexicana* complex cannot reliably be done with serological techniques but must be done by direct demonstration of the parasite.

For diagnosis of leishmaniosis in dogs, serological methods, such as the indirect fluorescent-antibody test, ELISA, and Western blotting (14), or in vitro cultivation (9) is of great value. In areas where the disease is endemic, however, the usefulness of serological methods is limited to epidemiological studies because there are infected dogs that do not show a specific antibody reaction (4, 28).

Direct detection of the parasites is best achieved by in vitro multiplication of the promastigote form. This method was found to be more sensitive than microscopic examination of stained biopsy preparations (12, 25, 27) and has replaced animal experimentation (hamster infection). Moreover, in vitro cultivation allows further characterization of the isolates by isoenzyme analysis (29) and could gain significance in examination for resistance to chemotherapeutic agents (16). However, diagnostic in vitro cultivation is cumbersome (because it includes preparation of complex media and time-consuming preparation of blood-containing samples) and expensive. Cultivation for diagnostic purposes takes in general 3 to 4 days and in few cases up to 10 days until positive results are obtained. Most of our diagnostic samples are negative for *Leishmania* spp., and these samples in particular need extensive microscopic examination. Furthermore, the routine handling and cultivation of leukocyte fractions from AIDS patients require special precautions. Successful in vitro cultivation needs fresh material and is limited not only by microbial contamination (see, e.g., reference 22) but also by the existence of members of this genus that are difficult to culture (23).

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TABLE 1. Clinical specimens

Host	Material	No. of patients/no. of samples	
		Proven cases ^a	Nonproven cases ^b
Human	Skin biopsy	6/10	19/21
	Bone marrow biopsy	4/5	23/25
	Whole blood ^c	6/11	24/24
Dog	Lymph node aspirate	15/16	3/3
	Whole blood ^d	12/13	

^a Leishmaniosis was confirmed by cultivation and PCR.

^b Specimens from subjects for whom a positive result never was obtained with any method (including serological methods).

^c Supplemented with heparin.

^d Supplemented with EDTA.

An alternative approach to parasitological methods is identification by using molecular biological techniques such as specific DNA probes (19) or the PCR. Several *Leishmania* DNA targets have been identified, and their values for use in a PCR-based diagnosis were partially evaluated. However, all tests described hitherto are specific either for cutaneous leishmaniosis (6–8, 22, 30) or for the visceral disease (18, 27). In order to cover all clinical samples sent to our institute with a single test, we evaluated primers that were selected from the sequence of the small-subunit (SSU) rRNA gene (33), which is repeated more than 100 times in the *Leishmania* genome (32, 33).

In this study we compared the PCR as a diagnostic method with in vitro cultivation to detect *Leishmania* spp. in samples from humans and dogs with a tentative diagnosis of leishmaniosis.

MATERIALS AND METHODS

Clinical samples. The samples that were available for this study are listed in Table 1. For routine parasitological diagnosis we received 96 samples from 76 patients returning to Switzerland from areas where leishmaniosis is endemic; 25 patients had suspicious cutaneous symptoms, and 51 had a tentative diagnosis of visceral leishmaniosis (43 HIV-positive patients and 8 with unknown HIV status). The samples consisted of 31 skin biopsies, 30 bone marrow biopsies, and 35 anticoagulated (with heparin) blood specimens. Biopsy material was collected aseptically in several Swiss clinics and sent to our institute in transport medium (culture medium as described below) supplemented with 100 IU of heparin (Liquemin; Roche, Basel, Switzerland) per ml.

In addition, lymph node aspirates from 18 dogs returning from the Mediterranean area with clinical symptoms of leishmaniosis were available. From 12 of these dogs we also obtained anticoagulated (with EDTA) blood.

Biopsy material was washed by centrifugation (300 × g, 10 min) with Hanks' balanced salt solution without calcium and magnesium. Originally the material was cut to small pieces with a sterile scalpel; later it was mechanically homogenized by being passed through a sterile metal sieve (width of mesh, 0.4 mm) with the help of the plunger of a disposable 2-ml syringe.

Anticoagulated blood (10 to 20 ml) was centrifuged (400 × g, 40 min) in 10-ml plastic tubes, and the buffy coat was carefully removed and resuspended in 6 ml of Hanks' balanced salt solution. The leukocytes from this suspensions as well as from bone marrow biopsies were further separated from the remaining erythrocytes by centrifugation (400 × g, 40 min) over Ficoll-Paque (Pharmacia, Uppsala, Sweden; density, 1.077 g/ml) and were subsequently washed twice by centrifugation (300 × g, 10 min) in Hanks' balanced salt solution.

The preparations were divided into four samples, with two being used for cultivation and two for PCR.

From the original whole blood samples (supplemented with either heparin or EDTA), two additional aliquots of 500 µl were set aside for PCR analysis.

About half of all specimens tested were collected before the PCR was introduced. The aliquots destined for PCR analysis were stored at –20°C. The other specimens were processed consecutively and were usually subjected to PCR as single samples.

In vitro cultivation. Cultivation was done basically as described earlier (9) with a complex culture medium (5) supplemented with 25 mg of gentamicin per liter and 15% fetal calf serum; the pH was adjusted to 7.2.

Cultivation was done in tissue culture tubes with a flattened side (Nunc; Nunc Denmark; volume, 10 ml), allowing direct examination of the culture with

an inverted microscope. Cultivation was performed at 27°C without a gas phase. The medium was changed only if microbial contamination was detected by microscopy. Microscopic examination started at day 2 and was done every other day until day 10 at longest.

Specimen processing and DNA extraction. DNA extraction was performed on either fresh or frozen (–20°C) material by digestion with proteinase K (100 µg/ml, 1 h, 56°C) directly in PCR buffer (see below). The enzyme was inactivated by incubating the tubes at 96°C for 10 min. All sample preparations were done in a volume of 100 µl from which 10 and 25 µl (from whole blood) were used for DNA amplification.

Proteinase K digestion with 500 µl of whole blood or with samples containing blood was done after lysis of the erythrocytes in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) as described by Sauvaigo et al. (31).

Biopsy material was treated as described above, except for skin biopsies, which were incubated for 2 h with 200 µg of proteinase K per ml.

DNA amplification reaction. PCR was performed in a Perkin-Elmer thermal cycler. The reaction mixture (100 µl) contained PCR buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 2.5 mM MgCl₂, 0.5% Tween 20), 200 µM each deoxyribonucleotide, 1 µM each primer, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). The "hot start" technique was used, and 35 cycles of 94°C for 60 s (denaturation), 60°C for 60 s (annealing), and 72°C for 60 s (extension) were performed. The primers used were primers 221 and 332 as designated by van Eys et al. (33), giving rise to a 603-bp fragment upon amplification. Amplicon production was determined by agarose gel electrophoresis (1.5%) with 20 µl of the reaction mixtures.

All specimens were tested in duplicate, with the second reaction being spiked with 10⁶ copies of the plasmid bearing the control target (see below). The specimens were considered inhibitory if the internal control was not visible or was only barely visible by gel electrophoresis. PCR then was repeated with a 100-times-diluted sample as well as with an aliquot after phenol-chloroform extraction and ethanol precipitation. A negative control with no DNA added was included in all tests.

Cross-contamination problems were avoided by following good laboratory practice, including the use of aerosol-guarded tips, and by performing the DNA extractions in a laminar flow hood which subsequently was irradiated by UV light. The areas used for DNA isolation, PCR, and amplicon analysis were strictly separated.

Molecular cloning. The cloned target sequence (vector pKS) was cleaved with *NarI*, which recognizes a unique internal restriction site, and was ligated to a fragment (about 100 bp) obtained after *HpaII* digestion of vector DNA. The resulting construct yields an amplicon of about 700 bp upon amplification with primers 221 and 332 and is easily discriminated from the *Leishmania*-specific product of 603 bp.

DNA concentrations were measured fluorometrically (TKO 100 fluorometer; Hoefer, San Francisco, Calif.).

RESULTS

In order to test the specificity of the chosen primers, we performed a database (GenBank/EMBL, release 39) search with the Genetics Computer Group (University of Wisconsin) program installed on a Crimson computer. The primer pair matches perfectly with all of the known sequences of the SSU rRNA genes of medically important *Leishmania* species. This was confirmed by performing PCR with DNA from one isolate each of the following six species by using promastigote stages from in vitro cultures (data not shown): *L. infantum* LEM75, *L. donovani* DD8 (World Health Organization [WHO] reference strain), *L. tropica* K27 (WHO reference strain), *L. aethiopica* L100 (WHO reference strain), *L. braziliensis* LEM781, and *L. major* LEM769.

The database search also revealed that amplification of an rDNA fragment might occur with lower kinetoplastids that parasitize invertebrate hosts (*Leptomonas* sp., *Endotrypanum* sp., and *Crithidia fasciculata*). They have sequences identical to that of the upstream primer (primer 221) and one (*Leptomonas* sp.) or two (*Endotrypanum* sp. and *C. fasciculata*) mismatches with the downstream primer (primer 332). No other target sequences that could give rise to an amplification product with these two primers were found.

The results of PCR and in vitro cultivation with a variety of diagnostic samples from humans and dogs are summarized in Table 2. All in all, PCR and in vitro cultivation showed identical results with 94 of 95 human samples (blood leukocyte fractions, bone marrow biopsies, and skin biopsies). With all 70

TABLE 2. Diagnosis of leishmaniosis in specimens from humans and dogs by PCR and in vitro cultivation

Host	Material	No. positive/total no.		
		Proven cases ^a		Nonproven cases ^b (PCR)
		PCR	Culture	
Human	Skin biopsy	6/10	7/10	0/21
	Bone marrow biopsy	5/5	5/5	0/25
	Blood			
	Whole	8/11	ND ^c	0/24
	Leukocyte fraction	7/11	7/11	0/24
Dog	Lymph node aspirate	16/16	16/16	0/3
	Blood			
	Whole	5/13	ND	ND
	Leukocyte fraction	3/3	1/3	ND

^a Leishmaniosis was confirmed by cultivation and PCR.

^b Specimens from subjects for whom a positive result was never obtained with any method (including serological methods).

^c ND, not done.

diagnostic samples from nonproven cases, no false-positive results were obtained (Table 2). Thus, the specificity of PCR reached 100%.

Cutaneous leishmaniosis. Of 31 skin biopsies from 25 patients, 10 samples were from 6 patients eventually proven to be infected with *Leishmania* spp. (two of these isolates were characterized by isoenzyme analysis at the WHO Collaborating Center, Montpellier, France, as *L. braziliensis*). All diagnostic samples were tested with both methods, and for six samples PCR and in vitro cultivation detected the infection. However, an additional sample confirmed by cultivation was negative by PCR. A subsequent PCR done with genomic DNA of this cultured isolate proved to be positive. Thus, sensitivities were 60 and 70% for PCR and in vitro cultivation, respectively.

From one patient with a proven case of cutaneous leishmaniosis we obtained three subsequent samples (one biopsy before and one aspirate and one biopsy during chemotherapy with ketoconazole). Only the last biopsy specimen was positive by both methods.

Visceral leishmaniosis. Bone marrow biopsies were obtained from 27 patients with suspicious visceral leishmaniosis. In all five samples from four AIDS patients with proven visceral leishmaniosis, the infection was detected by PCR and in vitro cultivation (sensitivity, 100%). One isolate was characterized by isoenzyme analysis at the WHO Collaborating Center (Montpellier, France) as *L. infantum*.

For identification of circulating *Leishmania* organisms in the blood, heparinized blood was used for PCR and the leukocyte fraction after Ficoll separation was used for PCR and in vitro cultivation. The PCR results for the 35 whole blood samples correlated with the results of PCR and culture of the corresponding leukocyte fractions in 34 cases (7 positive and 27 negative results). From one sample from an AIDS patient with a proven *Leishmania* infection (by PCR and cultivation from a bone marrow biopsy obtained a short time before), PCR was positive with whole blood whereas both PCR and culture were negative with the leukocyte fraction obtained after Ficoll purification. Two earlier blood samples from the same AIDS patient (who at that time was considered to be infected because of seropositivity in ELISA with promastigote antigen) were negative in both tests.

Canine leishmaniosis. All dog-derived lymph node aspirates showed identical results with PCR and in vitro cultivation (16

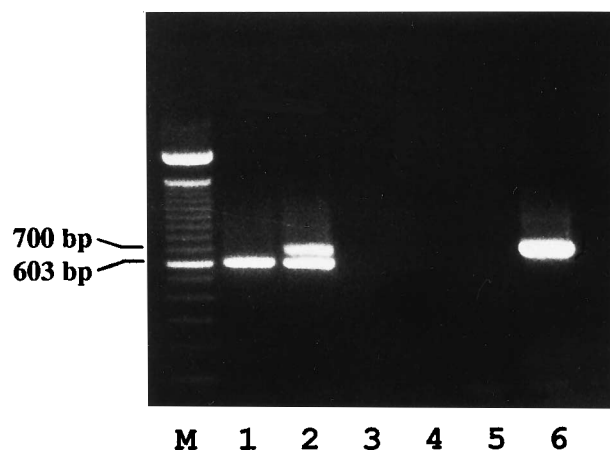


FIG. 1. Detection of amplification products after gel electrophoresis and ethidium bromide staining. The 603-bp fragment corresponds to *Leishmania* amplified DNA, and the 700-bp fragment corresponds to internal control amplified DNA. Inhibition of PCR is detected by abolished amplification of the control target in the parallel amplification reaction. Lanes: M, 100-bp marker; 1, sample 1 (positive); 2, sample 1 spiked with internal control; 3, sample 2 (inconclusive); 4, sample 2 spiked with internal control (inhibition); 5, no DNA template; 6, internal control alone.

positive and 3 negative; one of these dogs was later diagnosed to be infected with *Hepatozoon canis*, and leishmaniosis was excluded in the other two cases). The sensitivity of the PCR with dog lymph node aspirates was 100% compared with cultivation. Of 13 samples from dogs with proven leishmaniosis, 5 also were positive in PCR with DNA obtained from whole blood. The corresponding cultivation with the leukocyte fraction was done only in three of the PCR-positive cases, with one being positive.

Detection of PCR inhibition. In order to detect samples containing substances inhibitory to PCR, we coamplified an internal control target in a parallel reaction (Fig. 1); we detected inhibition of the PCR in 18 samples (12 human skin biopsies, 3 samples of whole blood, 2 bone marrow biopsies, and 1 dog lymph node aspirate). Conclusive results (2 positive and 16 negative) could be obtained after amplification reactions with 100-fold-diluted samples or with the samples after extractions with organosolvents.

DISCUSSION

In this study we have applied PCR to amplify a fragment of the SSU rRNA gene to detect *Leishmania* spp. in a variety of clinical samples and have compared PCR with diagnostic in vitro cultivation. The specificity of the primers was evaluated both theoretically and empirically: a computer assisted database search revealed that the primers are specific for medically important species of *Leishmania* and for a few kinetoplastids that parasitize arthropods. No false-positive results were obtained with 70 clinical specimens from suspected but nonproven cases. Thus, we conclude that the primers specifically detect *Leishmania* spp. in specimens from vertebrate hosts. We then performed PCR with DNAs of six medically important, defined isolates of *Leishmania*, and they all gave the diagnostic fragment. This confirms and extends the range of defined *Leishmania* isolates tested (33) with this primer pair.

The results of PCR and in vitro cultivation showed an excellent congruence. Only one discrepancy, a false-negative PCR result with a skin biopsy, was observed. PCR with DNA of this cultured isolate proved positive. Therefore, the reason

for the failure of the PCR is not that this isolate represents a genetic variant unsuitable for amplification with the chosen primer pair. Rather, an unequal apportionment of the original sample is most likely the reason for this false-negative PCR result.

The sensitivity of parasite detection in skin biopsies is known to be influenced by several factors, such as the stage of the lesion (active or healing) and the uneven distribution of amastigotes in the lesion tissue (25). Moreover, severe microbial coinfection of lesions may impair diagnostic in vitro cultivation (22). The sensitivity of our cultivation from skin biopsies was 70%, which is comparable to the findings of other groups, who reported sensitivities of between 56 and 67% (8, 22, 25). Studies performed in areas where cutaneous leishmaniasis is endemic found sensitivities for PCR (with minicircle kinetoplastid DNA as a target) of 80% with 30 patients (22) and 85% with 13 patients (8), which are distinctly higher than our finding of 60% (10 specimens). However, de Bruijn et al. (8) found in a follow-up study a reduced sensitivity of their PCR assay (57.6% with 68 patients).

The sensitivities of both PCR and cultivation were 100% with bone marrow biopsies and with lymph node aspirates from dogs. Comparable results were obtained by other groups (18, 27), with sensitivities of 82% (bone marrow biopsies) and 85% (spleen biopsies). The sensitivity of cultivation in one of these studies (27) was only 55% with a simpler medium. Thus, our cultivation system seems to be optimal.

Detection of the parasites in blood samples of immunodeficient patients (AIDS patients) was possible but had a lower sensitivity of 64%. This is in good agreement with the findings of Hassan et al. (18). Using a PCR specific for *L. donovani*, they detected *Leishmania* DNA in blood samples from 5 of 11 proven cases (sensitivity of 45%). Despite its moderate sensitivity, this noninvasive blood PCR assay may be used as an initial test, reducing the need for bone marrow biopsies and especially to follow the parasitemia during treatment. However, more clinical experience is needed for this approach.

The detection of amastigote stages in blood from dogs has been thought to be without diagnostic relevance, as Abranches et al. (1) could not detect parasites in blood smears from 23 dogs with leishmaniasis. In our study, PCR detected leishmanial DNA in 5 of 13 blood samples from dogs with leishmaniasis that were positive in ELISA for specific immunoglobulin G. Only one of three PCR-positive cases proved positive by cultivation.

In the future it might be of interest to correlate the parasitemia with the immune status of the host. In our study, for example, two blood samples from an HIV-positive patient with a strong specific antibody reaction in ELISA but without clinical symptoms were negative as tested by PCR but became positive after the onset of clinical signs. Another AIDS patient without detectable specific antibodies in ELISA and without typical clinical signs of leishmaniasis showed a parasitemia that was readily detected by both methods used.

PCR with whole blood detected one additional case of leishmaniasis in a human and two additional cases in dogs compared with PCR and culture with leukocyte fractions after Ficoll purification. This indicates that leukocyte separation might be critical. This is especially true, in our experience, if the blood samples are processed more than 24 h after collection. PCR detected *Leishmania* organisms in the sediment (erythrocyte fraction) of the Ficoll purification of two tested samples. Hence, *Leishmania* parasites present in blood samples may be lost with the Ficoll procedure. We then also tried to prepare the blood sample for cultivation in the same way as we do for PCR (lysis of the erythrocytes in TE buffer) but

found that microscopic detection of the parasites was difficult because of a large amount of cell debris remaining in the culture.

Inhibition of the PCR as detected by the abolished amplification of the internal control target was obvious in 18 samples. Inhibition in blood samples or in samples containing blood may be due to the presence of residual amounts of hemoglobin. With skin biopsies, 12 of 31 samples showed inhibition in PCR. Dilution of the samples (100-fold) as well as extraction with organosolvents and DNA precipitation enabled us to overcome this problem. Therefore, with skin biopsy material it might be advisable to perform PCR with both an undiluted sample and a diluted sample at once.

The diagnosis of leishmaniasis is currently genus specific, with in vitro cultivation giving the opportunity to identify the species by isoenzyme analysis after mass cultivation. The PCR fragment obtained in our assay allows the differentiation of *L. donovani* from all other species upon restriction enzyme analysis (33). Further improvement may be achieved by analyzing this fragment by single-strand conformation polymorphism or by denaturing gradient gel electrophoresis.

The diagnostic PCR described in this work yields a unique product (besides the inevitable primer artifacts) of 603 bp, in contrast to other approaches, in which unspecific side products appear. Therefore, this approach has the advantage that results are easily and unequivocally interpreted as analyzed after agarose gel electrophoresis. Furthermore, a high level of sensitivity is guaranteed, as there is no competition of unspecific products for components of the PCR.

We have shown that PCR with primers derived from the *Leishmania* SSU rRNA gene is a reliable test to detect the parasite in humans and dogs with a tentative diagnosis of leishmaniasis. The assay is both rapid and simple, as it involves preparing DNA from a variety of clinical specimens (whole blood, bone marrow, skin biopsies, and lymph node aspirates) in PCR buffer directly and using this preparation for DNA amplification without any further purification steps. Furthermore, it is complete because it includes an internal target which detects inhibition of the reaction and thus excludes false-negative results. Results are obtained by agarose gel electrophoresis, which is the simplest and the most common way to detect amplicons. PCR results are available within 24 h, compared with 3 to 10 days for culture. This assay proved to be feasible as a routine diagnostic test in our laboratory.

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